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Transcriptomic analysis of responses to infectious salmon anemia virus infection in macrophage-like cells

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ABSTRACT

The aquatic orthomyxovirus infectious salmon anemia virus (ISAV) is an important pathogen for salmonid aquaculture, however little is known about protective and pathological host responses to infection. We have investigated intracellular responses during cytopathic ISAV infection in the macrophage-like Atlantic salmon kidney (ASK) cell line by microarray analysis (1.8k SFA2.0 immunochip) and a functional assay for glutathione. Gene transcription changed rapidly and consistently with time and with minor differences between two virus isolates. While several pro-inflammatory and antiviral immune genes were induced, genes involved in cell signaling and integrity were down-regulated, suggesting isolation of infected cells from cell-to-cell interaction and responses to external signals. Differential expression of genes regulating cell cycle and apoptosis implied opposite cues from host cell and virus. This was in pace with massive down-regulation of genes involved in biosynthesis and processing of nucleotides and nucleic acids. Significant down-regulation of several genes involved in metabolism of reactive oxygen species suggested increased oxidative stress, which was confirmed by a functional assay showing reduced levels of glutathione during infection. Testing of expression data against a microarray database containing diverse experiments revealed candidate marker genes for ISAV infection. Our findings provide novel insight into cellular host responses and determinants for acute cytopathic ISAV infection.

1. Introduction

The infectious salmon anemia virus (ISAV) is an aquatic orthomyxovirus (genus *Isavirus*) causing high mortalities in aquaculture worldwide. Although improved control management has reduced the number of outbreaks, it is still considered a major threat due to high risks of emergence of new pathogenic strains and asymptomatic infections in feral fish. ISAV causes a multisystemic disease characterized by high mortality with exophthalmia, pale gills, severe anemia, decreased hematocrit value, ascites and severe hemorrhagic necrosis of internal organs (reviewed in Kibenge et al., 2004; Rimstad and Mjaaland, 2002). The structural characteristics and viral binding and entry of ISAV are well described but the molecular and functional understanding of the host–pathogen interaction needs further investigation. Several ISAV isolates with different virulence properties and pathogenicity *in vivo* are described (Kibenge et al., 2006; Mjaaland et al., 2005). Mjaaland et al. (2005) classified 12 isolates as acute or protracted variants, and survival and virus clearance correlated with the ability to induce a proliferative cellular immune response. However, *in vitro* correlation between replication properties of different ISAV isolates and the development of cytopathic effect (CPE) (Mjaaland et al., 2002) is less clear. Naturally occurring aquatic viruses will likely not benefit from high virulence since chances of spreading will be minor due to the low density of potential hosts. Thus, it has been proposed that emergence of high-virulent ISAV strains is a fairly recent event promoted by intense farming with high stock densities. This could imply that resistance of fish is determined by a complex interaction of immunological and physiological factors, making it difficult to dissect direct effects of infection from the secondary changes. Therefore, *in vitro* studies are important for understanding mechanisms of pathogenesis and resistance to ISAV at the cellular level. ISAV replicates in leukocytic and endothelial-like cells in all organs, but viral loads are highest in heart, spleen and head kidney (Falk and Dannevig, 1995; Falk et al., 1995; Jørgensen et al., 2007b; Moneke et al., 2003, 2005a,b). Several salmon cell lines permissive for ISAV have been described,

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most of them of macrophage-like origin (Dannevig et al., 1995, 1997; Rolland et al., 2002; Wergeland and Jakobsen, 2001). The Atlantic salmon kidney (ASK) cell line derived from head kidney has proven highly susceptible to ISAV, producing more definitive CPE compared to related cell lines (e.g. SHK-1, TO) (Jørgensen et al., 2006b; Rolland et al., 2005). Based on its ability to adapt to standard cell culture routines it is commonly used for growth and diagnosis of ISAV (B. Dannevig, National Veterinary Institute, pers. comm.). Apart from studies addressing transcriptional responses of immune-related genes (Jensen and Robertsen, 2002; Jørgensen et al., 2006b, 2007a; Kileng et al., 2007; Koppang et al., 1999), few have investigated the effect of ISAV on general cellular processes. Joseph et al. (2004) investigated ISAV-induced apoptosis in other commonly used Atlantic salmon cell lines, SHK-1 and TO, and found that CPE was due to virus-induced apoptosis in SHK-1 but not in TO.

The purpose of this study was to investigate protective mechanisms induced by host cells and pathological alterations caused by ISAV at the transcriptomic level by the use of microarray analysis, which is regarded as one of the most promising approaches for systemic insight into cellular processes. We used a high-density salmonid fish cDNA microarray (SFA2.0 immunochip) designed for studies of responses to pathogens and stressors. The previous version (SFA1.0, GEO GPL1212) has been used to assess responses to pathogens and inflammatory agents (MacKenzie et al., 2006a,b, 2008), handling stress (Krasnov et al., 2005a) and toxicity (Koskinen et al., 2004; Krasnov et al., 2005b) in rainbow trout, developmental disturbances in Baltic salmon (Vuori et al., 2006) and toxicity in brown trout (Krasnov et al., 2007; Merilainen et al., 2007). The updated 1.8k platform (SFA2.0, GEO GPL6154) has substantially improved coverage of immune genes, and has been employed to analyze gene expression in ISAV-infected salmon (Jørgensen et al., 2008). Our findings shed new light on the molecular basis of pathogenesis of cytopathic ISAV infection in a common target cell-type, and may provide foundation for further insight into virulence factors and potential protective host responses.

2. Materials and methods

2.1. Cells and virus

Atlantic salmon kidney cells were kindly provided by B. Krossøy (Department of Fisheries and Marine Biology, University of Bergen, Norway). Passages 40–70 were routinely split and cultured in L-15 medium (Cambrex Bio Sciences, Verviers, Belgium) supplemented with 50 μg/ml gentamicin, 4 mM L-glutamine, 40 μM β-mercaptoethanol and 10% fetal calf serum. Cells were infected at MOI = 1 with ISAV isolates I2 (Mjaaland et al., 2005) and I4 (Glesvaer 2/90 strain). Infection was performed at 15 °C in 75 cm^2 culture flasks as described (Jørgensen et al., 2006b), and cells were harvested at 1, 3, and 5 days post-infection (DPI) for immediate RNA isolation. Immunolabeling of virus-positive cells was performed as described (Mikalsen et al., 2005) using an anti-ISAV monoclonal antibody 3H6F8 (Falk et al., 1998). Counterstaining with the nuclear stain 4,6-diamino-2-phenylindole (DAPI) was performed using the ProLong Gold reagent containing DAPI (Molecular Probes, Invitrogen) according to manufacturer's instructions. For virus quantification, see Section 2.4.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted and purified using combined Trizol/PureLink micro-midi kit (Invitrogen, CA, USA), quantified by Nanodrop (Thermo Fisher Scientific, USA) and checked by gel electrophoresis. Samples for qPCR were DNAse-treated using Turbo DNA-*free*™ (Ambion, TX, USA) according to manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using random hexamer primers and TaqMan® Reverse Transcription reagents (Applied Biosystems, CA, USA) according to manufacturer's protocol.

2.3. Microarray analysis

The SFA2.0 immunochip includes 1800 unique clones printed in six spot replicates. The genes were selected by their functional roles and the platform is enriched in a number of functional classes, such as immune response (236 genes), cell communication (291 genes), signal transduction (245 genes) and receptor activity (126 genes), apoptosis (120 genes), cell cycle (76 genes), protein catabolism (90 genes) and folding (70 genes) and response to oxidative stress (39 genes). The gene composition and sequences are provided in GEO (GPL6154). Control and test samples (10 μg RNA in each) were labeled with respectively Cy3-dUTP and Cy5-dUTP (Amersham Biosciences, UK) using the SuperScript™ Indirect cDNA Labeling System (Invitrogen). The cDNA synthesis was performed at 46 °C for 3 h in a 20 μl reaction volume, followed by RNA degradation with 0.2 M NaOH at 37 °C for 15 min and alkaline neutralization with 0.6 M hepes buffer. Labeled cDNA was purified with Microcon YM-30 (Millipore, MA, USA). The slides were pretreated with 1% BSA fraction V, 5× SSC, 0.1% SDS (30 min at 50 °C) and washed with 2× SSC (3 min) and 0.2× SSC (3 min) and hybridized overnight at 60 °C in a cocktail containing 1.3× Denhardt's, 3× SSC, 0.3% SDS, 0.67 μg/μl polyadenylate and 1.4 μg/μl yeast tRNA. After hybridization slides were washed at room temperature in 0.5 SSC and 0.1% SDS (15 min), 0.5 SSC and 0.01% SDS (15 min), and twice in 0.06 SSC (3 min each). Scanning was performed with GSI Lumonics ScanArray 4000 (Packard Biosciences/PerkinElmer) and images were processed with GenePix 6.0 (Axon Instruments). Low-quality spots marked by GenePix were excluded from the analyses. After subtraction of background and normalization with Lowess, the differential expression was assessed by difference of the mean log-ER (expression ratio) from zero (six spot replicates of each gene, Student's *t*-test). Complete data from results are provided in GEO (GSE11160).

Functional analysis of statistically significant gene expression changes was performed with Ingenuity Pathways Analysis (Ingenuity Systems). This software analyses RNA expression data in the context of known biological response and regulatory networks as well as other higher order response pathways. Genes with expression changes more than twofold ($P < 0.01$) at 5 DPI were considered for analysis. The significance of the association between the data set and the canonical pathway is measured in two ways: (1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed. (2) Fischer's exact test is used to calculate a *P*-value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone. All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base.

2.4. Quantitative real-time RT-PCR (qPCR)

Primers for qPCR (Table S1) were designed using Primer Express 2.0 (Applied Biosystems). PCR amplicons and specificity were confirmed by gel electrophoresis. qPCR assays were optimized using 2× SYBR® Green Master Mix (Applied Biosystems) and varying template and primer concentrations. Primers for ISAV segment 7 and 18S rRNA (reference gene) were as described (Jørgensen et al., 2006b). Amplification efficiencies (*E*) were calculated from 10-fold

serial dilution of cDNA templates according to Rasmussen (2001) and inter/intra-assay reproducibility was confirmed on selected samples. Optimal conditions in a 25 μl reaction were 3 μl 1:10 cDNA (1:1000 for 18S rRNA) and 0.4–0.6 μM of each primer. PCR was performed in triplicates on ABI Prism 7000 Sequence Detection System (Applied Biosystems), and run protocol was according to manufacturer's recommendations. Data were analyzed according to the ABI Prism 7000 Sequence Detection System user manual and relative expression calculated by the $\Delta\Delta C_T$ method adjusted for E (Pfaffl, 2006). Based on a previous validation (Jørgensen et al., 2006a) and testing by BestKeeper (Pfaffl, 2001) and GeNorm (Vandesompele et al., 2002) on the present material, only 18S rRNA among four tested was qualified as a reference gene.

To quantify virus production, RNA was isolated from serial dilutions of 1 ml supernatants containing known amounts of ISAV I4 (by titration and determination of infective dose, $TCID_{50}$/ml) and cDNA produced as described (Section 2.2). qPCR was performed and C_T plotted against virus titre to obtain a standard curve for virus. Using this standard curve on cellular RNA (both genomic and mRNA) will therefore give a signal that corresponds to the amount of RNA in viral particles (only genomic from both infectious and noninfectious particles).

2.5. GSH assay

The amount of reduced glutathione in mock- and ISAV-infected (I2 and I4) ASK cells on 1, 3 and 5 DPI was measured using an ApoGSH glutathione kit (BioVision Inc., CA, USA). Cells were trypsinized, collected by centrifugation and further processed for GSH measurement according to manufacturer's protocol. GSH level in each sample was adjusted for protein concentration determined on aliquots using the BCA Protein Assay Kit (Pierce, IL, USA). Statistical analysis was performed by ANOVA/Neuman–Keul's test and Student's t-test for unpaired data ($P < 0.05$). Data are presented as means of three independent experiments ± SE (standard error).

3. Results

3.1. Viral infection

To examine if ISAV isolates induced different transcriptomic responses, ASK cells were infected with two isolates, I2 and I4, having different high-polymorphic region (HPR) genotypes but inducing similar acute disease forms in experimental challenge. Infection at MOI = 1 ensured a high ratio of virus particles to cells, as was confirmed by immunofluorescence staining of virus showing that >98% of cells were positive for both isolates after 3 days post-infection (Fig. 1a). A slight decrease in the number of viable cells already at 5 DPI could indicate onset of CPE, which was significant after 8–9 days (data not shown). RNA levels of segments 7 (encoding the non-structural NS protein) also increased rapidly for both isolates throughout infection as confirmed by quantitative real-time PCR (Fig. 1b).

3.2. Transcriptomic analysis

To construct an overall picture of the transcriptomic changes we selected 376 genes with differential expression ($P < 0.01$) in at least one sample. The number of differentially expressed genes increased consistently with time (Fig. 2a). High correlation between gene expression profiles in all samples (Pearson's, $r = 0.67 \pm 0.02$) was evidence for the consistency of transcriptomic responses. Cluster analyses divided samples by time; the samples from cells infected with I2 and I4 were linked pair-wise by the time-points (Fig. 2b). Different clustering procedures produced identical results (data not shown). To facilitate the presentation and interpretation of results, differentially expressed genes were arranged by their structural and functional roles with special attention to genes involved in antiviral- and immune-related responses (Fig. 3). Such divisions are usually problematic due to pleiotropy; many genes can be implicated in different processes and thus the functional groups may overlap to a large extent. Nonetheless, strong and consistent changes were prominent in genes involved in the following processes; cell signaling and integrity (cytoskeleton structure and organization), cell proliferation and nucleotide biosynthesis, and cell death and ROS metabolism. Importantly, each group included several genes with an early response, which was observed already at 1 DPI (Fig. 3).

3.3. Immune-related genes (across groups)

Regulated genes with well-described roles in immunity were found in all groups (Fig. 3, marked *) and their expression changed in both directions. Microarray expression levels were confirmed by qPCR analysis on selected genes (Fig. 4). Several immediate-induced genes were indirectly implicated in pro-inflammatory responses through interleukin-1 (IL-1) signaling. The phosphotyrosine-independent ligand for the SH2 domain of tyrosine kinase Lck *p62* (also known as *sequestome-1*) is involved in regulation of NF-κB activation in response to IL-1β (Sanz et al., 2000) but also other processes such as activation of T cells (Isakov and Biesinger, 2000). In addition, this protein contains an UBA domain that binds poly-ubiquitin chains helping proteasomal degradation of ubiquitinated proteins (Seibenhener et al., 2007). *Pyrin* has been linked to the newly characterized inflammasome (Yu et al., 2006) which activation ultimately induces an IL-1β response (Petrilli et al., 2007). Tax (human T-cell leukemia virus type 1)-binding protein also known as TRAF6-interacting protein is an IL-1 signal transducer (Ling and Goeddel, 2000). Other up-regulated immune-related genes were *beta-2-microglobulin* (involved in expression of MHC class I molecules) and *nf-kb inhibitor alpha* (*nfkbia*), an inhibitor of the pleiotropic mediator of immune and inflammatory responses, NF-κB. Up-regulation of lectin genes, in particular galectin-like genes (putative homologs to *galectin-1* and *-9*), was a marked feature of responses to ISAV. Galectins are small, highly conserved proteins that bind to various ligands with strong preference to sugars, especially galactose. Induction of galectins by IFN (Imaizumi et al., 2002) and double-stranded RNA (Imaizumi et al., 2007) was reported in mammals, and they are implicated in regulation of diverse immune functions (Liu, 2005). The galectins as well as one gene encoding an uncharacterized tripartite motif (TRIM) protein, a member of the multi-gene family with an important role in antiviral defense (reviewed in Towers, 2007), also showed strong responses to VHSV, a rhabdovirus causing viral hemorrhagic septicemia in rainbow trout (O'Farrell et al., 2002). Several immune-related genes were down-regulated and these were mainly implicated in cell signaling and communication (*cytokine B14*/*cxcl14*-like; receptors for complement, TNF and cytotoxic cells). Heat shock protein 60 kDa is involved in toll-like receptor-mediated (Zanin-Zhorov et al., 2006) and integrin-mediated (Barazi et al., 2002) antiviral responses.

3.4. Cell signaling and integrity

Coordinated down-regulation of genes with regulatory roles in cell surface and extracellular-associated processes was a remarkable feature of ASK responses to ISAV, and in addition to the immune-related genes mentioned above, many other genes from this group showed rapid expression changes. Decreased mRNA levels of genes encoding secretory regulatory proteins CYR61, con-

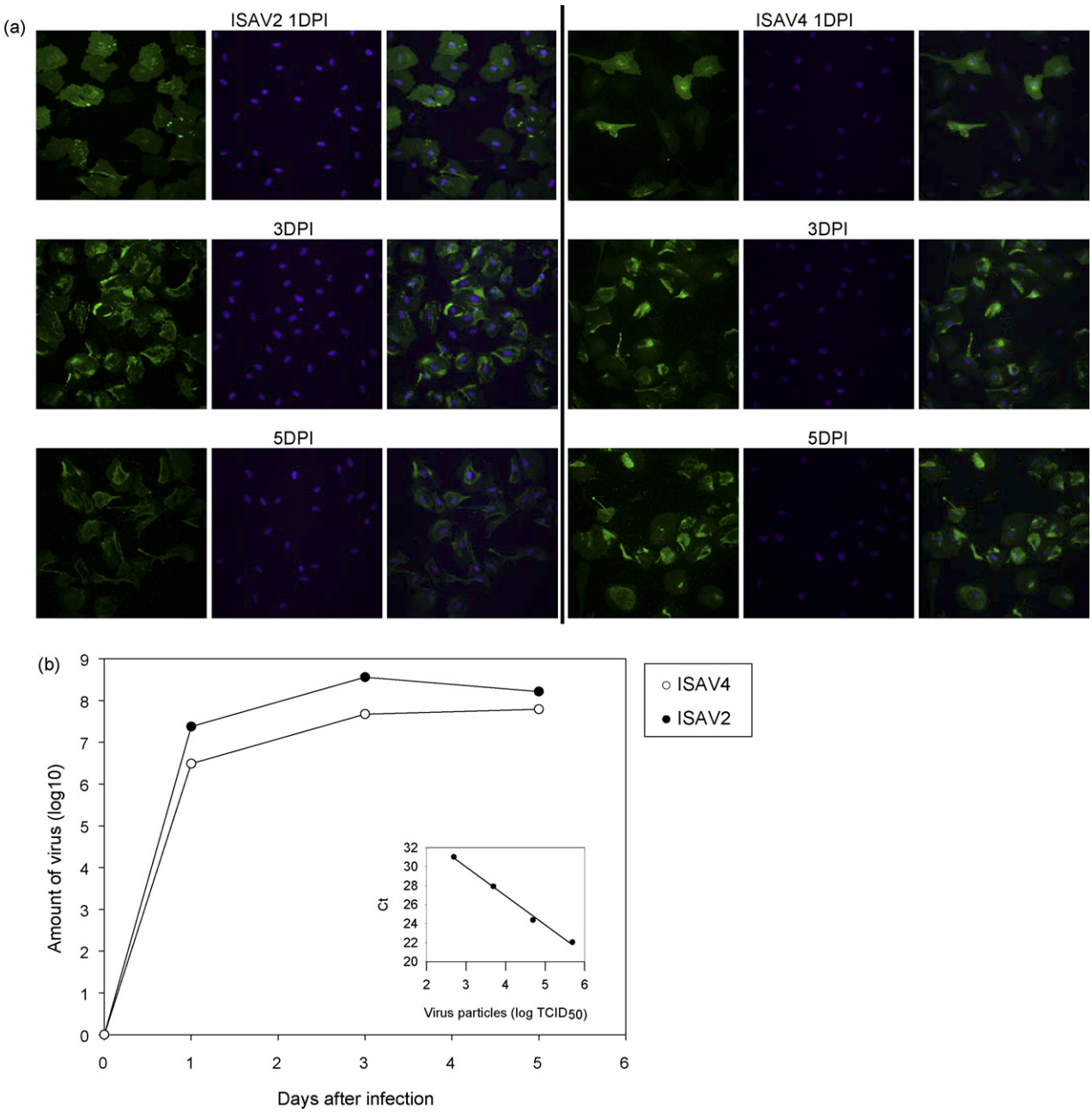

Fig. 1. Kinetics of ISAV replication in ASK cells. (a) Immunofluorescence staining of I2 and I4 at 1, 3 and 5 DPI using an anti-HE mAb and nuclear staining (DAPI). (b) Quantitative real-time PCR of ISAV segment 7 given as log(viral particles) based on a standard curve obtained from ASK culture supernatants containing known titres of I4 (see Section 2).

nective tissue growth factor, glucose-6-phosphate isomerase (a glycolytic enzyme with cytokine activity) as well as fibronectin (a cell-surface protein binding various compounds) and its receptor, was evidence for a decline in cell signaling and communication. Up-regulation of *tissue factor pathway inhibitor 2* could contribute to suppressed chemoattraction (Kopp et al., 2004). We could also predict changes in lipid composition and the electric properties of membranes (decrease in *delta-6 fatty acid desaturase* and *high-density lipoprotein* (*HDL*)*-binding protein*, *Na*/*K ATPase* and *voltage-dependent anion-selective channel*) as well as in contacts between the cell surface and cytoskeleton (decrease in *HDL-binding protein* and *annexin 6*). *HDL-binding protein* was also one of the most down-regulated genes in this survey. Known as the salmon homolog of *vigilin*, this RNA-binding protein can form complex with the IFN-inducible double-stranded RNA-specific adenosine deaminase ADAR1 (Wang et al., 2005) which is one of the antiviral effector proteins within the complex innate immune response (Jayan and Casey, 2002). Thus, down-regulation of *vigilin* may compromise the RNA-editing capacity of ADAR1 and could therefore enhance viral replication.

3.5. Cell proliferation and nucleotide biosynthesis

Consistent with the large-scale expression changes in cell surface and extracellular space components, we observed a rapid induction of genes that link external changes to regulation of the key cellular functions such as cell proliferation and protein biosynthesis. A putative homolog to G1/S-specific *cyclin D2* was rapidly and strongly up-regulated. The D-type cyclins play a key part in coupling of extracellular cues with the regulation of cell cycle (Sherr,

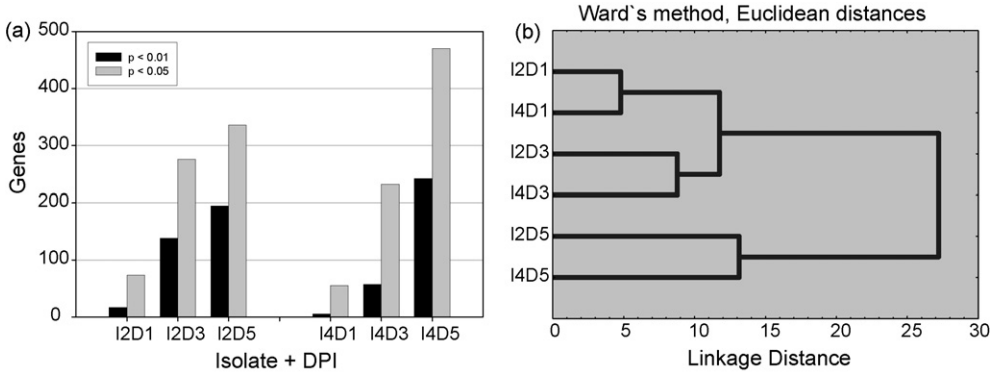

Fig. 2. Overall transcriptomic responses in ISAV-infected ASK cells. (a) Number of genes with significant differential expression for I2 and I4 at different *P*-values (*t*-test, $P < 0.05$ and <0.01). (b) Hierarchical clustering of samples by Euclidian distances with Ward's method.

1995). These proteins govern entrance and progression through G1 phase via phosphorylation of antiproliferative retinoblastoma protein (reviewed in David-Pfeuty, 2006). A similar expression profile was seen in one more regulator of the G1 phase, *tyrosine-protein kinase FRK*, which has been associated with both stimulation (Fresno Vara et al., 2001) and suppression (Cabodi et al., 2000) of cell proliferation. Among genes with reduced expression were both positive (*cyclin G*) and negative (*p53*, *btg-1*) regulators of proliferation. Reduced expression was also observed for genes involved in biosynthesis of nucleotides and regulation of transcription at different levels; *histone deacetylase 1* (partner of *p53*) and *high mobility group protein 2* (*hmgb2*) both modify the organization of chromosomes, and a homolog of *cofactor for Sp1 activation* (encoding CRSP-complex subunit 7) is required for the activation of transcriptional initiation by RNA polymerase II. Recent publications have also revealed an essential role for histone deacetylase in transcription of IFN-responsive genes (Nusinzon and Horvath, 2003). Genes for heterogeneous nuclear riboproteins and pre-mRNA splicing factor RNA helicase are important for processing of transcripts, while *fibrillarin* takes part in the first step of processing of ribosomal RNA. Reduced expression of genes involved in maturation of RNA was concordant with down-regulation of the translation machinery (ribosomal proteins, translation initiation and elongation factors) plus chaperones that mediate folding and transport of newly synthesized proteins (heat shock proteins, *t-complex protein* and *ubiquitin*).

3.6. Cell death and ROS metabolism

In contrast to the observed reduction in protein biosynthesis, genes encoding mitochondrial proteins involved in production of ATP (cytochrome *c* and *-b* and subunits of cytochrome *c* oxidase) as well as protoporphyrinogen oxidase, encoding a protein integrated in the mitochondrial membrane, were up-regulated. Release of cytochrome *c* from mitochondria could also mediate a pro-apoptotic signaling pathway through subsequent activation of caspases. A gene homologous to *cyclophilin-40* (*cypD*) was down-regulated, it encodes a peptidyl prolyl *cis*,*trans*-isomerase (PPIase) and component of the permeability transition pore involved in the mitochondrial pathway of apoptosis (Lin and Lechleiter, 2002). Interestingly, *cypD* was similarly regulated in highly pathogenic influenza infections in contrast to persistent infections (Kash et al., 2004). Several other genes with potential apoptosis-inducing activities were also down-regulated, including *tumor suppressor p53*, *serine/threonine-protein kinase 17A*, *histone deacetylase 1*, *coronin-1* (partner of *p53*) and *transaldolase*. We also observed regulation of genes directly or indirectly involved in metabolism of reactive oxygen species (ROS). Activation of *ferritin* in conjunction with down-regulation of *transaldolase* might indicate reduced production of ROS. Ferritin binds intracellular iron, which catalyzes generation of free radicals. Transaldolase, the key enzyme of the pentose phosphate pathway provides NADPH, which is rate-limiting for the mitochondrial production of ROS; overexpression of this gene enhances oxidative stress (Banki et al., 1998). However, coordinated down-regulation of several genes crucial for maintaining reducing conditions within cells (*superoxide dismutase*, *glutathione reductase*, *selenide water dikinase* and *sulfotransferase*) suggested a reduced redox power and developing oxidative stress. In this respect it is noteworthy to mention up-regulation of *c-jun*, a pro-apoptotic transcription factor activated with ROS via the immune-related JNK pathway (reviewed in Shen and Liu, 2006). Furthermore, extended pathway analysis of genes changed more than twofold ($P < 0.01$) with Ingenuity Pathways Analysis (see Section 2) revealed that the most significantly activated signaling pathway was the Nrf2-mediated oxidative stress response pathway (Supplementary Fig. S1). The Nrf2 transcription factor binds antioxidant response elements (AREs) and activates a number of genes involved in cellular responses to oxidative stress (Ishii et al., 2000). To further substantiate these findings we tested a functional assay for measurement of intracellular glutathione (GSH), a cysteine containing tripeptide and prominent mediator of antioxidant defense in eukaryotic cells. ASK cells were infected with I2 and I4 and levels of GSH measured in cell lysates at 1, 3 and 5 DPI (Fig. 5). Mock-infected and staurosporine-treated (only at 1 DPI) cells served as negative and positive controls, respectively. Both isolates showed a clear trend of elevated GSH levels after 1 day with subsequent reduction towards the last time-point in three replicate experiments. However, only I2 induced a significant GSH reduction at days 3 and 5 compared to day 1. Taken together, these results showed no clear regulation of cell death but increased oxidative stress that was inversely correlated with replication of ISAV.

3.7. Identification of ISAV marker genes

A powerful way to exploit microarray data is to compare data sets from several experiments representing different traits in order to identify candidate marker genes. We tested expression profiles from this study and data from a similar ISAV-challenge *in vivo* (heart, liver, gill and spleen) (Jørgensen et al., 2008) against data from other microarray experiments in our database (e.g. responses to toxicity, parasites, viruses, developmental disturbances, administration of hormones and handling- and feeding-related stress). Despite substantial differences between

Genes	I2D1	I2D3	I2D5	I4D1	I4D3	I4D5
Cell signaling and integrity						
*Galectin like 1	1.59	2.13	2.25	1.78	2.59	3.71
*Galectin-9 (VHSV-induced protein)-1	1.83	2.83	9.09	ND	2.65	8.95
*Selectin L-like	1.38	1.56	1.62	1.29	1.37	3.31
*Tripartite motif	2.31	4.75	3.68	2.80	7.24	12.59
*Ligand for the Lck SH2 domain p62	1.89	3.38	3.79	NS	1.94	2.84
*Beta-2-microglobulin-2	NS	2.36	3.30	NS	1.58	1.66
Tissue factor pathway inhibitor 2 precursor	1.66	1.47	NS	NS	1.80	2.18
*Pyrin-1	NS	1.88	1.84	NS	2.53	3.93
*Tax-1 binding protein	1.17	1.58	2.18	ND	2.15	2.80
78 kDa glucose-regulated protein precursor	1.68	2.30	2.54	NS	1.67	NS
CYR61 protein	NS	-2.08	-4.00	NS	-1.31	-1.88
*Small inducible cytokine B14 precursor	-1.57	-2.84	-5.53	NS	-2.03	-2.94
Connective tissue growth factor	-1.86	-1.56	-2.72	NS	-1.43	-1.94
Glucose-6-phosphate isomerase-1	-1.41	-1.83	-6.03	-1.41	-1.50	-2.80
*Fibronectin precursor	-2.44	-3.47	-34.17	-1.29	-2.40	-7.00
*Fibronectin receptor beta	NS	-1.80	-3.55	NS	-1.76	-3.12
*Tumour necrosis factor receptor	NS	-1.82	-2.86	NS	-1.86	-2.05
*Nonspecific cytotoxic cell receptor protein-1	NS	-1.75	-5.49	NS	-2.36	-4.94
*Complement receptor 1-1	NS	-1.37	-2.75	NS	-1.65	-1.93
*B-cell receptor-associated (prohibitin)	NS	-1.36	-1.92	NS	-1.66	-2.00
*Sphingosine 1-phosphate receptor Edg-3	-1.48	-1.77	-2.60	-1.49	-2.34	-6.57
Na/K ATPase alpha subunit-2	-1.28	-1.55	-1.89	-1.24	-1.50	-1.84
Voltage-dependent anion-selective channel	NS	-1.28	-1.95	NS	-1.64	-2.25
Delta-6 fatty acid desaturase	NS	-1.61	-2.29	-1.20	-1.56	-3.27
F-actin capping protein alpha-1 subunit	-1.31	-1.80	-2.45	-1.33	-1.52	-2.17
Cofilin, muscle isoform	NS	-1.80	-2.73	NS	-1.61	-2.04
Thymosin beta-4-2	NS	-1.51	-2.75	NS	-1.44	-1.65
Keratin, type I cytoskeletal 9	NS	-1.71	-2.85	NS	-1.64	-1.93
Annexin 6	NS	-2.03	-4.08	NS	-1.72	-4.19
PDZ and LIM domain protein 1	-1.38	-2.09	-4.41	NS	-2.10	-2.68
High density lipoprotein-binding protein	-1.54	-2.98	-11.46	-1.69	NS	-4.69
Cell proliferation and nucleotide biosynthesis						
G1/S-specific cyclin D2	1.82	2.36	5.81	1.97	4.29	10.89
Tyrosine-protein kinase FRK	1.97	2.40	ND	ND	2.97	6.11
*NF-kappaB inhibitor alpha-3	NS	2.10	2.56	NS	1.85	4.23
Ubiquitin	NS	2.26	1.83	NS	1.61	1.59
Cyclin G1	-1.34	-1.38	-1.75	-1.29	-1.69	NS
Ribonucleoside-diphosphate reductase	-2.36	-2.65	-3.34	-1.40	-1.34	-2.20
High mobility group protein 2	NS	-1.59	-2.74	NS	-1.70	-2.23
BTG-1	NS	-1.32	-1.63	NS	-1.64	-2.02
Cofactor required for Sp1 activation	NS	-1.90	-3.06	NS	-1.46	-1.41
Heterogenous nuclear ribonucleoprotein U	NS	-1.65	-2.22	NS	-1.88	-3.77
Heterogeneous nuclear ribonucleoprotein A1-2	NS	-1.92	-2.36	NS	-1.38	-1.54
Putative pre-mRNA splicing factor RNA helicase	NS	-1.53	-2.92	NS	NS	-2.13
Fibrillarin	NS	-1.52	-1.76	NS	-1.69	-1.61
Bifunctional aminoacyl-tRNA synthetase	-1.70	-2.04	-4.02	NS	-1.54	-2.66
60S ribosomal protein L5-1	-1.43	-2.25	-4.03	-1.34	-2.05	-4.63
40S ribosomal protein S6	NS	-1.28	-1.60	NS	-1.33	-1.78
Translation initiation factor 3 subunit 5	NS	-1.13	-1.59	-1.45	-1.68	-2.97
Translation initiation factor 3 subunit 6-1	NS	-1.91	-2.92	-1.34	NS	-1.61
Translation elongation factor 1 alpha 3	NS	-1.99	-2.22	NS	-1.58	-1.54
Translation elongation factor 1 alpha 1	NS	-1.96	-2.55	-1.41	-1.72	-2.02
Elongation factor 1-alpha 2	NS	-1.85	-3.51	NS	-1.81	-2.69
Heat shock 70kDa protein 8	-1.31	-1.75	-1.68	-1.24	-1.52	-2.07
*60 kDa heat shock protein-2	-1.74	-1.84	-1.72	-1.48	NS	-2.27
Heat shock cognate 71 kDa protein	NS	-1.90	-1.77	NS	-1.33	-1.42
Heat shock 70 kDa protein 1	-1.39	-2.06	-2.67	-1.24	-1.66	-2.71
T-complex protein 1, subunit 5	NS	-2.20	-2.55	NS	-1.80	-2.81
Cell death and ROS metabolism						
C-Jun protein	ND	2.18	2.96	ND	1.65	2.50
Protoporphyrinogen oxidase	1.56	1.62	2.10	1.54	1.80	3.37
Cytochrome c oxidase subunit I-2	NS	1.94	2.08	NS	NS	1.78
Cytochrome c oxidase subunit II	1.52	1.90	1.87	NS	1.40	2.06
Cytochrome c-1	1.25	1.67	2.49	NS	NS	2.99
Cytochrome b-3	NS	1.90	2.09	NS	NS	1.67
Ferritin H-3	NS	2.12	1.90	NS	1.68	1.89
Cyclophilin-40	NS	-2.11	-2.78	NS	-2.41	-4.90
Tumor suppressor p53	-1.53	-1.78	-2.25	NS	NS	-2.12
Histone deacetylase 1	-1.26	-2.10	-3.50	NS	NS	-4.89
Transaldolase	-1.40	-3.61	-8.13	NS	-2.25	-11.77
Serine/threonine-protein kinase 17A	NS	-2.37	-3.00	NS	-2.09	-1.85
Coronin-1C	-1.69	-3.59	-5.87	-1.49	NS	-5.61
Superoxide dismutase [Cu-Zn]	NS	-1.29	-1.49	-1.77	-1.66	-4.21
Glutathione reductase, mitochondrial-2	NS	-2.40	-9.12	NS	NS	-3.96
Selenide water dikinase 2-1	NS	-1.62	-2.34	NS	NS	-1.95
Sulfotransferase 4	NS	-1.91	-4.36	NS	-2.10	-4.70
N-myc downstream regulated protein-1	NS	-1.32	-3.30	NS	NS	-2.75

Fig. 3. Differentially expressed genes during acute ISAV infection *in vitro*. Macrophage-like ASK cells were infected with two cytopathic ISAV isolates, I2 and I4, and gene expression changes were assessed by microarray analysis after 1, 3, and 5 days. Differentially expressed genes are arranged according to their functional roles (as referred to in Section 3) and up/down-regulated expression. Genes with known or predicted roles in immunity and/or responses to viruses are marked with asterisk. Expression ratios of up- and down-regulated genes are in pink and blue, respectively, yellow means not significant and ND means not detected. Data are differential expression ratios to controls ($P < 0.05$ in at least three of six samples).

Table 1
Candidate marker genes for acute ISAV infection

Genes	*In vitro*[a]	*In vivo*[b]	All samples[c]	P-value[d]
Annexin 6	4[e]/6[f]	6/8	4/83	0.0000
PDZ and LIM domain protein 1	4/6	7/8	11/182	0.0000
Sphingosine 1-phosphate receptor Edg-3	3/6	7/8	11/167	0.0000
Complement receptor 1-1	3/6	5/8	2/82	0.0000
Tyrosine-protein kinase FRK	3/6	8/8	20/178	0.0001
Tissue factor pathway inhibitor	3/6	7/8	16/173	0.0001
Tripartite motif	5/6	6/8	9/82	0.0003
Galectin-1 like	5/6	8/8	13/83	0.0003
NF-κB inhibitor alpha-3	4/6	8/8	33/183	0.0006
CYR61 protein	3/6	3/8	2/81	0.0006
Fibronectin receptor beta	4/6	4/8	17/192	0.0007
Cofilin	4/6	8/8	40/203	0.0009
Small inducible cytokine B14	3/6	5/8	13/146	0.0010
Cyclophilin-40	4/6	3/8	15/181	0.0019
Serine/threonine-protein kinase 17A	3/6	5/8	22/202	0.0019
Na/K ATPase alpha subunit 2	3/6	5/8	23/209	0.0019
Pyrin-1	3/6	4/8	5/76	0.0025
Glutathione reductase, mitochondrial-2	3/6	4/8	7/85	0.0043
Keratin, type I cytoskeletal 9	4/6	5/8	30/182	0.0055
Heterogeneous nuclear ribonucleoprotein U	3/6	4/8	24/201	0.0084
B-cell receptor-associated protein BAP37-1	3/6	5/8	11/85	0.0084
G1/S-specific cyclin D2	5/6	8/8	68/209	0.0100

[a] This study.
[b] Expression data from ISAV-infected tissues (Atlantic salmon).
[c] Database of samples analyzed with previous and current versions of the SFA microarray.
[d] Genes are ranked by significance of preferential response to ISAV (Fisher's exact probability).
[e] Number of samples with differential expression (*t*-test, $P < 0.01$).
[f] Total number of samples in which the respective gene was detected.

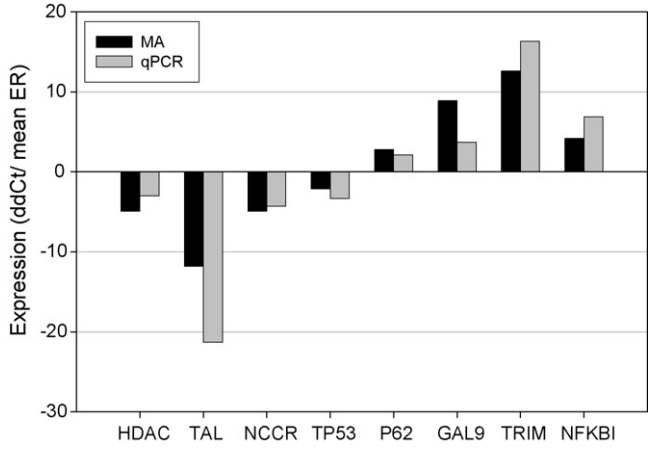

Fig. 4. Validation of microarray data by real-time qPCR on selected genes. Expression levels are given as $\Delta\Delta C_T$ (qPCR) or log-ER (MA). Genes included were as follows: *histone deactylase 1* (HDAC), *transaldolase* (TAL), *nonspecific cytotoxic cell receptor protein 1* (NCCR), *tumor suppressor p53* (TP53), *ligand for the Lck SH2 domain p62* (P62), *galectin-9* (GAL9), *tripartite motif* (TRIM), *nf-kb inhibitor alpha* NFKBI.

in vitro and *in vivo* data, a number of genes showed consistent responses to ISAV in both. Table 1 presents genes with differential expression in more than 50% of samples from ISAV-infected cells and tissues, which responded to virus with higher probability than to the other experiments. The proportions of samples with differential expression ($P < 0.01$) in ISAV experiments and our gene expression database (samples analyzed with SFA Versions 1.0 and 2.0) were compared with exact Fisher's probability test ($P < 0.01$). Importantly, these genes almost entirely belonged to the functional group that were most affected by virus infection (Fig. 3; genes associated with cytoskeleton, cell surface and extracellular space involved in cell signaling and integrity).

4. Discussion

As an initial approach to understand the cellular mechanisms explaining cytopathic ISAV infection, we investigated global transcriptomic changes using microarray in a common permissive salmon cell line. This methodology is particularly useful for elucidation of molecular pathologies of viruses in non-model species like salmonid fishes, where basic understanding of key cellular processes is scarce. The usefulness of *in vitro* models is determined by their ability to support viral replication and the biological relevance of the host–pathogen responses. We have investigated cellular responses in the macrophage-like ASK cell line, which is routinely being used for virus growth and diagnosis. Macrophages are also

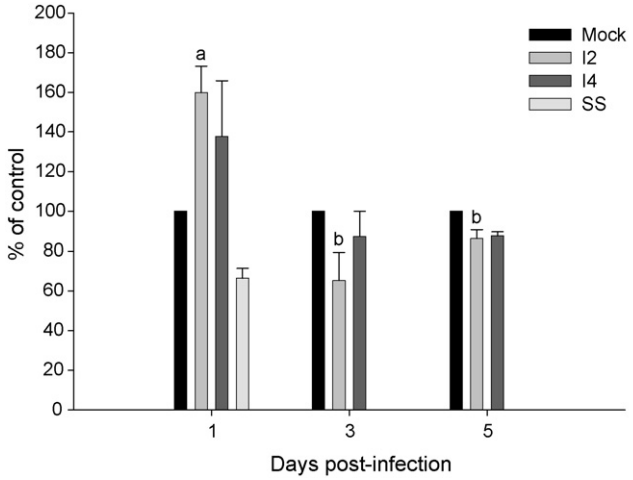

Fig. 5. Oxidative stress analyzed by levels of GSH in ISAV-infected ASK cells. Total GSH levels were measured in cells infected with cytopathic isolates I2 and I4 after 1, 3 and 5 days. Controls were mock-infected cells at all time-points (Mock) and cells treated with staurosporine (SS) for 24 h (positive control). Significantly different bars ($n = 3$, $P < 0.05$) are indicated by letters.

important target cells for ISAV *in vivo*. These cells mount a strong and rapid type I IFN phenotype following infection (Jørgensen et al., 2006b), which confers no protection (Kileng et al., 2007). Virus replication eventually causes CPE and cell death (Kibenge et al., 2006). We infected at high MOI which induced rapid and high levels of viral replication from day 1 until CPE, showing that ASK cells fully supported propagation of ISAV. This resulted in large-scale transcriptional changes which steadily increased throughout infection. All differentially expressed genes were either up- or down-regulated without any temporal fluctuations, suggesting that the observed changes were not random but true responses to infection (Fig. 3). Furthermore, the consistency of responses was confirmed by high correlation of the gene expression profiles in two independent experiments using two similar isolates. Relatively low similarity of transcriptomic responses to ISAV *in vitro* and *in vivo* (data not shown) does not confront the value of the *in vitro* model. Most changes observed in infected tissues are accounted for by processes that do not take place *in vitro*, e.g. interactions with the cellular and humoral immune factors and changes of cellular composition of tissues due to migration and differentiation of peripheral blood cells. A biological relevance implies meaningful changes that add to our understanding of host–pathogen interactions, and the important advantage of cell cultures is the possibility to outline primary, direct responses that are not obscured by complex systemic regulations.

Indeed, our results revealed a coordinated regulation of genes involved in key cellular processes. Among genes with known roles in immunity, up-regulation was mainly observed for genes involved in pro-inflammatory processes. Inhibition of NF-κB mediated activation inferred from up-regulated *nfkbia* could result in arrest or delay of important antiviral response pathways. However, this contradicts results showing that MHC I antigen presentation genes, as well as type I IFN, are rapidly induced in these cells (Jørgensen et al., 2006b). Interestingly, a similar induction of *nfkbia* is a marked feature of highly pathogenic influenza infection (Kash et al., 2004), where consequent loss of NF-κB activity was proposed to enhance cytotoxic effects of TNF and resulting cell death (Beg and Baltimore, 1996). Our observation that most immune-related genes implicated in cell signaling and integrity were down-regulated could imply isolation of cells from external cues. In this respect it is noteworthy to mention that in ISAV-infected salmon tissues we observed marked up-regulation of several immune-related signal transducers, e.g. *jak*, *STATs*, *FRK*, *junB* and *C/EBP* (Jørgensen et al., 2008). Induction of these pathways probably requires cellular interactions and/or other external stimuli. On the other hand, down-regulation observed in ASK cells could be due to arrest of host-gene transcription caused by selective production of viral mRNA.

The most prominent feature of ISAV infection was the co-occurrence of events that are unlikely to take place simultaneously under normal conditions. Early induction of *cyclin D2* that increased with time could indicate a forced entrance into cell cycle, since the only known role of this gene is suppression of negative regulation by retinoblastoma protein (David-Pfeuty, 2006). However, a number of changes implied that cells were unable to successfully continue and complete the cell cycle. Activation of the translation machinery is a key requirement for continuation of cell cycle (David-Pfeuty, 2006). However, we observed a massive down-regulation of translation/elongation factors and ribosomal proteins. This is a common feature of viral infections to facilitate selective translation of viral mRNAs. Inactivation of protein biosynthesis is also one of the well-known IFN-induced antiviral responses (Samuel, 2001). Decreased mRNA levels of the key enzymes for nucleotide biosynthesis should also negatively affect DNA replication. Furthermore, down-regulation was observed for *cyclin G* (regulator of cell cycle downstream from cyclin D2), *thymosin* (a regulator of actin involved in cytokinesis) and a panel of proteins implicated in the organization of chromosomes and maturation of mRNA. Cells that are unable to continue in the cell cycle are either arrested or eliminated via apoptosis. This decision is under control of *tumor suppressor p53*, which along with several other pro-apoptotic genes (*tnf receptor*, *serine/threonine-protein kinase 17A*, *histone deacetylase 1* and *coronin-1*), was down-regulated in ASK, adding to the uncertainty of the fate of cell death in these cells. The down-regulation of heat shock proteins may also increase viral survival, since release of HSPs from infected cells has been shown to bind TLRs on peripheral leucocytes and initiate an inflammatory response (Asea et al., 2000). Oxidative stress caused by enhanced production of ROS and reduced capacity for their neutralization is another important apoptotic pathway in virus-infected cells (Hasnain et al., 2003). ASK-infected cells showed up-regulation of the components of mitochondrial membrane and electron transport chain in conjunction with down-regulation of translational machinery, the major consumer of ATP, which collectively suggested enhanced production of ROS. Down-regulation of the proteins with scavenger capacities further increased the risk of oxidative stress. Among these, GSH is of particular interest since it has been recognised as a key contributor to the pathogenicity of numerous viral infections (Beck et al., 2000). We found an inverse correlation between the viral load and levels of GSH. Together with data from gene expression and pathway analysis, this finding suggested that oxidative stress is a prominent feature of acute cytopathic ISAV infection and may be an important determinant of cell death in ASK cells. From mammalian viruses it is well confirmed that virus replication is dependent on redox state, and that in particular levels of GSH explain viral persistence (Ciriolo et al., 1997; Garaci et al., 1997; Hennet et al., 1992; Nencioni et al., 2003; Palamara et al., 1995). Interestingly, transcriptomic analysis of avian and 1918 influenza infections revealed a similar deregulation of genes involved in glutathione metabolism as one of the prominent determinants for the highly pathogenic nature of these viruses (Kash et al., 2004, 2006). We do not know the mechanism of the GSH loss in ASK cells. Cells could be depleted of GSH by an oxidative process or through nonspecific leakage over the plasma membrane (e.g. due to membrane perturbation from virus fusion), as has been shown (Ciriolo et al., 1997).

We paid special attention to a set of candidate marker genes that responded rapidly to ISAV while being less sensitive to other pathogens and stressors (Table 1). The majority of these genes were implicated in immunity and in processes affected by viral infection such as cell signaling (signal transduction, binding and apoptosis) and integrity (modification of proteins, assembly of cytoskeleton and cell cycle control). It is necessary to keep in mind that interpretation of gene expression data should take into account co-existence of two conflicting programs in the infected cells. Altered regulation of genes may either indicate a role in host protection or its use by virus. Several genes with early induction encoded proteins with binding properties. Proteins involved in binding of sugar, such as galectins, could be used by viruses as illustrated by the fact that GAL-1 promotes adhesion of HIV-1 (Ouellet et al., 2005). However, conservation of responses to diverse viruses across large phylogenetic distances, from fish to mammals, points to possible important roles of GAL and tripartite motif genes in antiviral defense. Several members of the TRIM family are shown to detect viruses and restrict their replication (reviewed in Nisole et al., 2005). An interesting feature of these proteins in the context of applications related to aquaculture (i.e. disease resistance and selective breeding), is the observation that they can account for inter-species differences to HIV infectivity (Sokolskaja and Luban, 2006). Interestingly, human TRIM20 is a partner of *pyrin* (James et al., 2007), another up-regulated gene in ASK cells. At present there is no suf-

ficient evidence for the direct involvement of GAL in protection against viruses. However, this role was indicated by the discovery of an up-regulation of GAL by IFN and polyI:C, which is mediated by TLR3, phosphatidylinositol kinase (P13K) and interferon regulatory factor 3 (Imaizumi et al., 2007). Consequences of GAL overexpression are difficult to predict, given their extremely diverse and complex modulatory effects on the immune system. This can be illustrated with several examples. For instance, GAL-9 induces aggregation and death of T helper (T_H) cells resulting in selective loss of IFN-γ producing cells (Zhu et al., 2005). GAL-1 may increase apoptosis of lymphocytes (Rabinovich et al., 2002) and induce production of IL-10, which suppresses T_H1-type responses (van der Leij et al., 2004). However, the same protein was reported to augment survival of lymphocytes and secretion of cytokines (Levroney et al., 2005). GAL-9 works as a chemoattractant for eosinophils (Imaizumi et al., 2002) but GAL-1 inhibits attachment of T cells to extracellular matrix (Rabinovich et al., 1999). We believe that these marked responses of galectins and TRIM to fish viral infections will promote in-depth studies on these proteins in the future.

In conclusion, we have used transcriptomic profiling and functional assay support to elucidate cellular mechanisms explaining acute cytopathic ISAV infection in a macrophage-like cell line, which represents an important target cell-type *in vivo*. Our data provide new information on the roles of genes involved in inflammation, cellular proliferation and apoptosis and demonstrate for the first time that increased oxidative stress may be an important determinant for the cytopathic outcome of ISAV infection in these cells. These findings may contribute to improved disease control of this important aquaculture disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2008.04.019.

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